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**9827228.9**

10 DEC 1998

3. Full name, address and postcode of the or of each applicant (underline all surnames)

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Patents ADP number (if you know it) 773796001

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4. Title of the invention **CANCER DETECTION METHOD AND REAGENTS**

5. Name of your agent (if you have one)

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CANCER DETECTION METHOD AND REAGENTS

The present invention relates to highly sensitive and specific methods for detecting the presence of cancer marker proteins in the bodily fluids of a mammal, to autoantibodies for use in these methods, to immortalised cells and hybridomas for obtaining these autoantibodies and to kits for performing the methods. These methods are useful in the early detection of carcinogenic or pre-neoplastic modifications in asymptomatic patients, in monitoring the progress of cancer, in screening for recurrence of the disease in patients who have previously undergone anti-cancer treatment, in monitoring the efficacy of a systemic treatment in a patient and in determining the most appropriate treatment for a particular patient.

Cancer and pre-neoplastic cells are characterised by the production of cancer-associated marker proteins. These often consist of aberrant forms of wild-type proteins, which are produced by cancer cells as a result of genetic mutations or altered post-translational processing. Alternatively, cancer markers can also be proteins that become over-expressed in tumour cells, usually as a result of gene amplification or abnormal transcriptional regulation. In some cases, these two phenomena may occur at the same time leading to an accumulation of modified proteins throughout the development of the disease. For example, modified forms of Ras, p53, c-myc, MUC-1, c-erbB2 have been found to be associated with a wide variety of cancers.

Cancer-associated proteins are found both in the tissues and in the bodily fluids of an individual who carries pre-neoplastic or cancer cells. Their levels

are very low at the early stages of the carcinogenic process and increase during progression of the disease. The detection of these proteins has advantageously been used in routine tests for the diagnosis of cancer but, unfortunately, these assays have many limitations. In particular, commercial antibodies available for use in standard tests are usually not sensitive enough to detect the low levels of cancer-associated proteins that are found at the very early stages of the disease as, for example in asymptomatic patients, when a treatment would be the most effective. In addition, most commercial antibodies are not specific for modified forms of cancer-associated markers and cross-react with wild-type forms of these proteins. As a consequence, they are only useful for detecting substantial increases in serum levels of cancer marker proteins, which usually occur at advanced stages of cancer.

For example, the commercial assay CA15-3, which detects both unmodified and modified forms of MUC1, is useful in the diagnosis of metastatic breast cancers, which are characterised by elevated serum levels of MUC1. However, this assay cannot be used in screening for neoplasia or primary breast cancer because the serum levels of MUC1 at these stages do not differ significantly from those in normal individuals (Robertson et al. (1990), Eur. J. Cancer 26: 1127-1132). Other marker proteins such as, for example, carcinoembryonic antigen (CEA) and the marker CA19.9 have been reported to be elevated in the serum of patients with metastatic breast and colorectal cancer but not that of patients with primary cancers (Robertson et al. (1991), Cancer Immunol. Immunother. 133: 403-410; Thomas et al. (1991) Br. J. Cancer 63: 975-976). Also in the case of these cancer markers, available commercial assays are not able to

discriminate between modified and wild-type forms of the proteins and are therefore of limited use.

Furthermore, commercially available antibodies, by cross-reacting with normal forms of cancer-associated proteins, may also lead to false positive results. Thus, there is a need in the art for more sensitive and specific antibodies to use in these assays in order to detect pre-neoplastic and early carcinogenic modifications.

As used herein the terms "cancer-associated marker protein", "cancer-associated protein", "marker protein" or "cancer marker" all refer to cancer-associated modified forms of wild-type proteins.

Cancer markers often differ from the corresponding wild-type proteins in such a way that they are recognised as foreign molecules by the immune system of an individual, triggering an autoimmune-response. The immune-response may be humoral, leading to the production of autoantibodies against the cancer marker protein. Autoantibodies are naturally occurring antibodies directed to an antigen that an individual's immune system recognises as foreign even though that antigen actually originated in that individual. For example, modified forms of p53, MUC-1, c-myc, c-erb3 and Ras proteins may elicit production of autoantibodies. As used herein the term "autoantibody" refers to an antibody directed against a self-originating antigen, which antibody is naturally occurring in the circulation of an individual or to an antibody which exhibits the characteristics of the naturally occurring antibody in that it recognises the said self-originated antigen but which is produced outside the body, for example, by an immortalised cell-line or hybridoma.

35

As will be described in the Examples below, the

present inventors have surprisingly found that autoantibodies produced by patients suffering from cancer specifically recognise cancer-associated marker proteins from the same patients or from other patients with cancer and show very low cross-reactivity with wild-type forms of these proteins. Furthermore, the present inventors have found that the above autoantibodies have a much higher sensitivity than the antibodies currently used in routine tests and are therefore able to detect smaller quantities of cancer-associated marker proteins. Autoantibodies produced by patients with cancer may therefore be used to design alternative, more reliable and sensitive tests to detect pre-neoplastic or carcinogenic modifications in an individual from the very beginning of their occurrence. These assays may also be employed to detect cancer or pre-neoplasia in any other mammal, by utilising autoantibodies produced by a mammal from the same species as the one to be tested or autoantibodies having the same characteristics as such.

The present invention provides a more sensitive and specific assay system for the detection of pre-neoplasia or cancer in a mammal, which allows to detect cancer-associated marker proteins from the early stages of the disease.

Accordingly, in a first aspect the invention provides an *in vitro* method for detecting a cancer-associated marker protein present in a bodily fluid of a mammal which method comprises the steps of:

- (a) contacting a sample of bodily fluid from said mammal with antibodies directed against at least one epitope of said marker protein; and



(b) detecting the presence of any complexes formed between said antibodies and any marker protein present in said sample;

5 wherein said antibodies are mammalian autoantibodies to said cancer-associated marker protein which are  
~~derived from the same species as the mammal from which~~  
said sample has been obtained.

The presence of said complexes is indicative of  
10 the presence of cancer associated marker proteins in said mammal.

As used herein "derived" means an autoantibody or autoantibodies isolated from the said species or an autoantibody or autoantibodies having the  
15 characteristics of an autoantibody or autoantibodies isolated from said species.

The method of the invention may employ a single autoantibody directed against a particular cancer marker protein. Alternatively, a panel of  
20 autoantibodies recognising a number of cancer-associated proteins may be utilised in order to obtain a profile of cancer markers present in a particular individual. This leads to a more reliable diagnosis and provides information useful in the choice of the  
25 most appropriate treatment for an individual.

The assay method of the invention is performed on a sample of a biological fluid from the patient such as, for example, plasma, serum, whole blood, urine, lymph, faeces, cerebrospinal fluid or nipple aspirate,  
30 depending of the nature of the cancer to be detected. Since it is non-invasive the assay can be repeated as often as it is necessary to screen for early neoplastic or carcinogenic modifications, to follow the development of the disease, to test for recurrence  
35 of the disease, to verify the efficacy of a treatment or to select the most appropriate treatment for a

particular patient.

The method of the invention can be performed using any immunological technique known to those skilled in the art of immunochemistry. As examples, ELISA, radio immunoassays or similar techniques may be  
5 utilised. In general, an appropriate autoantibody is immobilised on a solid surface and the sample to be tested is brought into contact with the autoantibody. If the cancer marker protein recognised by the  
10 autoantibody is present in the sample, a complex autoantibody-marker protein is formed. The complex can then be detected or quantitatively measured using, for example, a labelled secondary antibody which specifically recognises an epitope of the marker  
15 protein. The secondary antibody may be labelled with biochemical markers such as, for example, horseradish peroxidase (HRP) or alkaline phosphatase (AP), and detection of the complex can be achieved by the addition of a substrate for the enzyme which generates  
20 a colorimetric, chemiluminescent or fluorescent product. Alternatively, the presence of the complex may be determined by addition of a marker protein labelled with a detectable label, for example an appropriate enzyme. In this case, the amount of  
25 enzymatic activity measured is inversely proportional to the quantity of complex formed and a negative control is needed as a reference to determine the presence of antigen in the sample. Another method for detecting the complex may utilise antibodies or  
30 antigens that have been labelled with radioisotopes followed by measure of radioactivity.

The method of the invention can be performed in a qualitative format, which determines the presence or absence of a cancer marker protein in the sample or in  
35 a quantitative format, which, in addition, provides a measurement of the quantity of cancer marker protein

present in the sample. The quantity of marker protein present in a sample may be calculated utilising any of the above described techniques. In this case, prior to performing the assay, it is necessary to draw a  
5 standard curve by measuring the signal obtained, using the same detection reaction that will be used for the assay, ~~from a series of standard samples containing~~ known concentrations of the cancer marker protein. The quantity of cancer marker present in a sample to be  
10 screened is then interpolated from the standard curve.

If it is necessary to verify the presence of a number of cancer marker proteins in a sample, the assay of the invention may be performed in a multi-well assay plate where each of the different  
15 autoantibodies utilised is placed in a different well.

The method of the invention can be employed in a variety of clinical situations such as, for example, in the assessment of the predisposition of an individual towards the development of a cancer, in the  
20 detection of pre-neoplastic or carcinogenic modifications in asymptomatic patients, in the diagnosis of primary or secondary cancer, in monitoring the progression of the disease in a patient, in screening for recurrence of carcinogenic  
25 modifications in a patient who has previously been diagnosed as carrying cancer cells and has undergone a therapy to reduce the number of these cells or in the choice of the more appropriate anti-cancer treatment for a patient suffering from cancer. The method of the  
30 invention is also suitable for veterinary use in the same clinical situations as the ones described above.

The assay method of the invention may be employed to detect cancer marker proteins that are associated with a variety of cancers such as, for example,  
35 lymphomas, leukaemias, breast cancers, colorectal cancers, lung cancers, pancreatic cancers, prostate

cancers, cervical cancers, ovarian cancers,  
endometrial cancers and cancers of the skin. The  
method of the invention is particularly suitable to  
detect and monitor primary breast cancer (PBC) and  
5 advanced breast cancer (ABC).

In a second aspect the invention provides  
monoclonal or polyclonal autoantibodies and reagents  
comprising said autoantibodies for use in the assay,  
10 which specifically recognise at least one epitope of a  
mammalian cancer-associated marker protein. Such  
autoantibodies may be isolated from the blood or  
peripheral blood monocytes of such a mammal,  
preferably a human. Alternatively, the autoantibodies  
15 can be produced by immortalised B lymphocytes or  
hybridomas and directed to an antigen originated in  
the mammal itself. Preferred autoantibodies to use in  
the assay include those against cancer-associated  
forms of the glycoprotein MUC1 (Batra, SK. et al.  
20 (1992) Int J. Pancreatol. 12: 271-283), the signal  
transduction/ cell cycle regulatory protein c-myc  
(Blackwood, E. M. et al. (1994) Molecular Biology of  
the Cell 5: 597-609), p53 (Matlashewski, G. et al.  
(1984) EMBO J. 3: 3257-3262), c-erb $\beta$ 2 (Dsouza, B. et  
25 al. (1993) Oncogene 8: 1797-1806) and Ras (Gnudi, L.  
et al. (1997) Mol. Endocrinol. 11: 67-76). However,  
autoantibodies against any other cancer-associated  
marker protein may be employed in the assay.  
Particularly suitable for the detection of breast  
30 cancers are autoantibodies against a modified MUC1,  
BRCA1, BRCA2, p53, c-myc, c-erb $\beta$ 2 or Ras protein  
associated with primary breast cancer and  
autoantibodies against a modified MUC1, BRCA1, BRCA2  
p53, c-myc, c-erb $\beta$ 2 or Ras protein associated with  
35 advanced breast cancer. These autoantibodies are  
preferably derived from patients diagnosed with the

same type of cancer as the one to which these cancer marker proteins are associated.

5 The invention also provides immortalised cell lines capable of producing the above autoantibodies. The cell lines of the invention may be produced by any  
10 ~~method known in the art. As will be described in~~ detail in Example 1 below, B cells from patients diagnosed with cancer may be, for example, immortalised with Epstein Barr Virus. ELISA or any similar techniques may be performed to screen for the production of autoantibodies, utilising marker proteins obtained from a patient affected from cancer which have been immobilised on a solid support.

15 In a further aspect the invention provides hybridomas capable of producing monoclonal autoantibodies against an epitope of a mammalian cancer associated marker protein, preferably a human  
20 cancer-associated marker protein. The hybridomas of the invention may be produced according to methods well known in the art (E. Harlow and D. Lane, "Antibodies: A laboratory Manual", pages 211-227, Cold Spring Harbor Laboratory, 1988).

25 The invention further provides kits for detecting one or more cancer-associated marker proteins in the biological fluids of a mammal. Such kits include at least mammalian autoantibodies directed against one or  
30 more epitopes of a cancer-associated marker protein and means for detecting the formation of complexes between the autoantibodies and the cancer-associated marker protein. Preferably, the autoantibodies are immobilised on a solid surface.

35 The present invention will be further understood with

reference to the following Examples and to the accompanying Figures in which:

- 5 Figure 1 shows the results of an ELISA assay to examine the reactivity of autoantibodies produced by B cells derived from six patients diagnosed with breast cancer (1 to 4, with primary breast cancer, 7 and 11 with advanced breast cancer). For each group of autoantibodies, MUC1 protein purified from the same patient from which the B cells were taken, from other patients or from normal subjects was used as immobilised antigens. The reactivity of mouse monoclonal B55 anti-MUC1 antibody in a parallel assay is included as a comparative control. PBS or antibodies produced by B lymphocytes derived from four healthy subject (N10, N12, N13 and N14) are used as negative controls. MUC1 was eluted from immunoaffinity columns using 0.25 M glycine pH 2.5.
- 10
- 15
- 20 Figure 2 shows the results of an ELISA assay to assess the reactivity of autoantibodies obtained from B cells derived from patients diagnosed with primary breast cancer with MUC1 protein from different sources. The reactivity of B55 is included as a comparative control. PBS is used as a negative control.
- 25
- 30 Figure 3 shows the results of a surface plasmon resonance experiment to measure the binding of autoantibodies produced by B cells derived from patients diagnosed with primary breast cancer to MUC1 protein isolated (a) from the serum of patients with advanced breast cancer or (b) from the urine of normal individuals.
- 35 Figure 4 shows the sequence of the peptide that was used to immunoaffinity-purify MUC1 antibodies from the

sera of patients with advanced breast cancer.

Figure 5 shows the results of an ELISA assay employing immobilised autoantibodies from a patient with (2) primary breast cancer or (3) advanced breast cancer to detect MUC1 protein purified from the serum of a patient diagnosed with advanced breast cancer or from the urine of a healthy individual. The result of a parallel assay utilising the anti-MUC1 C595 antibody (1) is included as a comparative example.

Figure 6 shows the results of an ELISA assay utilising immobilised autoantibodies from the B cells of patients with primary breast cancer to detect MUC1 protein in serum samples from healthy individuals or from patients diagnosed with primary or advanced

breast cancer. The results obtained with the C595 antibody in a parallel assay are included as comparative examples.

Figure 7 shows the results of an ELISA assay using immobilised autoantibodies from the B cells of patients with primary breast cancer to detect MUC1 protein in sequential serum samples from a patient with advanced breast cancer throughout the progression of the disease. The results obtained with the monoclonal C595 antibody in a parallel assay or with the commercial CA15-3 assay are included as comparative examples.

Example 1: Immortalisation of mononucleocytes.

Peripheral blood mononucleocytes were purified from a 4ml sample of heparinised blood from patients or normal individuals using lymphocyte separation medium

(ICN flow), as described in detail in the manufacturers instructions. Isolated mononucleocytes were washed in PBS and resuspended in 1 ml of a semipurified preparation of Epstein Barr Virus (EBV) from the B95-8 marmoset transformed leukocyte EBV-producing cell line. The cells were then incubated for 1 hour at 37°C in 5% CO<sub>2</sub> and centrifuged at 17000 rpm. The EBV supernatant was removed and the mononucleocytes were washed three times with RPMI medium, resuspended in RPMI medium supplemented with 10% fetal bovine serum and 5µg/ml phytohemagglutinin (PHA-P) and seeded in multi-wells tissue culture plates. The medium was changed every 3 days and used as a source of autoantibodies.

Example 2: Assessment of the reactivity of autoantibodies with MUC1 antigen from different sources

Methods:

1) Immunoaffinity purification of MUC1 antigen

MUC1 was purified from the serum of patients diagnosed with primary breast cancer or advanced breast cancer or from the urine of healthy subjects.

The mouse monoclonal B55 antibody (also known as NCRC 11 as described by Ellis et al. (1984) Histopathology 8: 501-516 and in International Patent Application No. WO 89/01153) was conjugated to CNBr sepharose beads. Serum or urine samples were diluted 1/10 in PBS and incubated with the antibody conjugated sepharose beads overnight at 4°C with rolling. The beads were centrifuged and the supernatant removed. In order to remove any molecule aspecifically bound to the beads, these were washed in PBS for 5 times or until the



washing buffer showed no absorbance at 280nm. Each wash was performed by resuspending the beads in PBS, rolling for 10 minutes, centrifuging and removing the supernatant. The washed beads were resuspended in 0.25 M glycine pH 2.5, rolled at room temperature for 10 minutes and centrifuged. The supernatant was removed, adjusted to pH 7 by addition of TRIS and stored at 4°C labelled "glycine fraction". The beads were then resuspended in 25 mM diethylamine (DEA) pH 11, rolled at room temperature for 10 minutes and centrifuged. The supernatant was again removed, adjusted to pH 7 by addition of TRIS and stored at 4°C labelled "25 DEA fraction". The beads were finally resuspended in 100mM DEA pH 11, rolled at room temperature for 10 minutes and centrifuged. The supernatant was removed, adjusted to pH 7 by addition of TRIS and stored at 4°C labelled "100 DEA fraction". The presence of MUC1 in the three fractions was confirmed by ELISA using the monoclonal antibody B55 or C595 (also known as NCRC, available from the Cancer Research Campaign). In order to remove contaminating immunoglobulins, fractions were incubated with DTT (to 50mM) for 30 minutes, then iodoacetamide (to 75mM) before being subjected to gel filtration on a S300 column. Fractions were assayed for MUC1 content by ELISA. MUC1 containing fractions are titrated so as to give equivalent absorbances to previous batches.

## 2) ELISA assay

Different MUC1 preparations, obtained as described above, were appropriately diluted with PBS and plated out at 50µl per well in a 96 well microtitre assay plate and left to dry overnight. The plate was then washed once with PBS/Tween to remove residual salt crystals, blocked for 60 minutes with a fresh solution of 2% (w/v) polyvinylpyrrolidone (PVP) in PBS and

washed three times with PBS/Tween. Culture supernatants of immortalised lymphocytes derived from patients diagnosed with primary or secondary breast cancer were plated out in triplicate, at 50 $\mu$ l per well. As a comparative control the mouse monoclonal anti-MUC1 antibody B55 was also plated in triplicate. The plate was incubated for 60 minutes at room temperature with shaking and washed four times with PBS/Tween. 50 $\mu$ l of HRP conjugated anti-human or anti-mouse secondary antibody (obtained from Dako) were added to each well at the dilution recommended by the manufacturer, and incubated for 60 minutes at room temperature with shaking. The plate was then washed again four times with PBS/Tween. 50 $\mu$ l of TetraMethylBenzidine (TMB) were added to each well and optical density (OD) at 650nm for each well of the assay plate was read kinetically over a period of 10 minutes.

Results:

Figure 1 shows the result of an ELISA assay to assess the reactivity of autoantibodies produced by lymphocytes derived from six patients diagnosed with breast cancer (1 to 4, with primary breast cancer, 7 and 11 with advanced breast cancer) with MUC1 protein purified from the same patient from which the antibody was taken, from other patients or from healthy subjects. The healthy subjects used in this study were women who had no clinal and/or mammographical evidence of breast cancer. The reactivity of the monoclonal anti-MUC1 B55 antibody was measured as a comparative control. Antibodies produced by lymphocytes from four healthy subjects (N10 to N14) were used as a negative control.

The results presented demonstrate that B lymphocytes derived from patients with breast cancer

produce autoantibodies that are able to recognise MUC1 protein isolated both from the same and from different patients. In addition, these autoantibodies bind with high specificity to MUC1 present in patients with cancer, showing almost no reactivity with MUC1 isolated from healthy individuals. These results are highly reproducible, since different autoantibodies show a very similar reactivity profile with MUC1 protein purified from different sources. Furthermore, the results obtained also indicate that the sensitivity of the autoantibodies for cancer-associated MUC1 is much greater than that observed for the monoclonal B55 antibody. Furthermore, antibodies produced by lymphocytes from normal patients did not show this profile.

Figure 2 shows the reactivity of autoantibodies secreted by immortalised B lymphocytes derived from patients with primary breast cancer with MUC1 protein from different sources, compared with that of B55. The profile of reactivity of the different autoantibodies is again very reproducible. The autoantibodies show high specificity for MUC1 present in the serum of patients with cancer and have almost no affinity for MUC1 isolated from healthy individuals or from the breast cancer cell line ZR75-1. Furthermore, the affinity of the autoantibodies for MUC1 protein associated with either primary breast cancer or advanced breast cancer is much higher than measured for B55.

Example 2: Measure of the affinity of autoantibodies with Surface Plasmon Resonance.

Methods

Surface Plasmon Resonance was performed on Iasys Biosensor Plus (from Affinity Sensor). MUC1 protein

from patients with advanced breast cancer and from normal individuals was adhered to amino silane coated cells following the manufacturers instructions and the cells were blocked with 1%(w/v)polyvynilpyrrolidone (PVP). Control cells coated only with 1% PVP were also produced. The binding of different dilutions of culture supernatants derived from B cells from patients with primary breast cancer was measured using the following experimental conditions:

10	Sampling interval:	0.3 msecs
	Stirrer speed:	70rpm
	Temperature:	24°C
	Binding Time:	3 min
15	Dissociation with PBS:	2 minutes
	Regeneration with 20mM Hcl:	3 minutes
	Re-equilibration with PBS:	5 minutes

## 20 Results

Figure 3 shows that the autoantibodies produced by B lymphocytes derived from a patient with primary breast cancer bind with a much higher affinity to MUC1 isolated from another patient with breast cancer than to MUC1 isolated from a healthy individual.

### Example 3: Detection of MUC1 antigen in ELISA assays utilising autoantibodies.

30

#### Method:

##### 1) Purification of anti-MUC1 autoantibodies from sera

The MUC1 peptide TAP2, with the sequence shown in Figure 4, was conjugated to CNBr-sepharose beads.

35 Pooled sera from patients diagnosed with advanced breast cancer were diluted 1/10 in PBS and were

incubated with the conjugated sepharose beads overnight at 4°C with rolling (in the ratio of 25ml of serum to 1ml of beads). After centrifugation the supernatant was removed and the beads were washed 5 times with PBS or until absorbance at 280nm was zero. Each wash was performed by resuspending the beads in PBS, rolling for 10 minutes, centrifuging and removing the supernatant. The beads were resuspended in 1ml of 3M sodium thiocyanate in PBS, rolled at room temperature for 10 minutes and centrifuged. The supernatant was removed and dialysed against PBS at 4°C. The anti-MUC1 content was then confirmed by ELISA using as immobilised antigen both MUC1 isolated from patients with advanced breast cancer and a MUC1 peptide, with sequence APDTRTPAPG and conjugated to BSA.

### 2) Biotinylation of anti-MUC1 autoantibodies

The autoantibodies obtained as described above were concentrated to a volume of 100µl by using centrifugal filters and then diluted to a volume of 1ml with 0.1 sodium tetraborate buffer pH 8.8. 20µg of N-hydroxysuccinimide biotin were added and the autoantibodies/biotin solution was incubated for 4 hours at room temperature with rolling. The reaction was stopped by addition of 10µl of 1M NH<sub>4</sub>Cl and incubation for ten minutes. The autoantibodies were then dialysed against PBS for thirty-six hours at 4°C to remove unbound biotin. Aliquots of the autoantibodies solution were frozen and stored at -20°C in the dark until use.

### 3) ELISA ASSAY

Culture supernatants of lymphocytes derived from patients with primary breast cancer or advanced breast cancer or the monoclonal anti-MUC1 C595 antibody were

plated out at 50 $\mu$ l per well in a 96 well microtrite assay plate and incubated overnight at 4°C. The plate was then washed 4 times with PBS/Tween, blocked for 60 minutes with a fresh solution of 2% (w/v) polyvinylpyrrolidone (PVP) in PBS and washed twice with PBS/Tween. 50 $\mu$ l per well of MUC1 from different sources were added. After incubation at room temperature for sixty minutes, the plate was washed again four times with PBS/Tween. 50 $\mu$ l of the appropriate biotinylated secondary antibody, either C595 or autoantibody purified from a pool of sera from a patient with advanced breast cancer, prepared as described above, were added to each well and incubated for 60 minutes at room temperature. After 4 washes with PBS/Tween, 50 $\mu$ l of streptavidin-HRP were added to each well and incubated at room temperature for 60 minutes. The plate was again washed four times, 50 $\mu$ l of TMB were added to each well and optical density (OD) at 650nm for each well of the assay plate was read kinetically over a period of 10 minutes.

#### Results:

Figure 5 shows the results of an ELISA assay utilising as immobilised antibodies autoantibodies produced by B lymphocytes derived from patients with primary or advanced breast cancer, compared with those obtained in a parallel assay with the monoclonal anti-MUC1 C595 antibody. The data indicate that autoantibodies from patients with breast cancer can be used in ELISA assays to specifically detect modified forms of MUC1 protein associated with cancer. These assays are more sensitive and show higher specificity than those utilising the monoclonal antibody C595.

Example 5: Use of the assay to detect MUC1 proteins in

serum samples of patients.

An ELISA assay was performed, as described in Example 4, on serum samples from healthy individuals or patients with primary or advanced breast cancer  
5 utilising as immobilised antibodies the autoantibodies produced by B lymphocytes derived from patients with primary breast cancer. A parallel assay utilising the the monoclonal anti-MUC1 antibody C595 was performed on the same samples. The results, shown in Figure 6,  
10 indicate that the assay employing autoantibodies is able to detect with high sensitivity MUC1 circulating in the blood of patients with breast cancer. In addition, contrary to utilising the monoclonal antibody C595, this assay has a very high specificity  
15 for cancer-associated forms of MUC1.

Example 6: Use of the assay to monitor the progression of the disease.

An ELISA assay was performed, as described in Example 4, on sequential serum samples from a patient  
20 diagnosed with metastatic breast cancer throughout the progression of the disease, using as immobilised antibodies the autoantibodies produced by B lymphocytes derived from patients with primary breast  
25 cancer or the monoclonal anti-MUC1 C595 antibody. The commercial assay CA15-3 was also performed on the same samples. Figure 7 shows that the assay employing autoantibodies can be used to follow the progression of cancer in a patient, wherein increasing levels of  
30 MUC1 detected in the assay indicate exacerbation of the disease. The data also demonstrate that the use of autoantibodies leads to results that better represent the development of the disease than those obtained with either the C595 antibody or the CA15-3 assay.

**CLAIMS:**

1. An *in vitro* method for detecting a cancer-associated marker protein present in a bodily fluid of a mammal which method comprises the steps of:

(a) contacting a sample of bodily fluid from said mammal with antibodies directed against at least one epitope of said marker protein; and

(b) detecting the presence of any complexes formed between said antibodies and any marker protein present in said sample;

wherein said antibodies are mammalian autoantibodies to said cancer-associated marker protein which are derived from the same species as the mammal from which said sample has been obtained.

2. A method as claimed in claim 1 wherein said sample is from a mammal substantially asymptomatic for pre-neoplasia or cancer.

3. A method as claimed in claim 1 wherein said sample is from a mammal symptomatic for cancer.

4. A method as claimed in claim 1 wherein said sample is from a mammal which has received therapy for cancer.

5. A method as claimed in any preceding claim wherein the mammal is a human and the autoantibodies are human autoantibodies.



6. A method as claimed in any preceding claim wherein said bodily fluid is plasma, serum, whole blood, urine, faeces, lymph, cerebrospinal fluid or nipple aspirate.

5

7. A method as claimed in any preceding claims wherein said cancer-associated marker protein is associated with lymphomas, leukaemias, breast cancers, colorectal cancers, lung cancers, pancreatic cancers, prostate cancers, cervical cancers, ovarian cancers, endometrial cancers or cancers of the skin.

8. A method as claimed in claim 7 wherein said cancer-associated marker protein is a breast cancer-associated marker protein.

15

9. A method as claimed in any preceding claims wherein said cancer-associated marker protein is a modified MUC1, BRCA1, BRCA2, p53, c-myc c-erb $\beta$ 2 or Ras protein.

20

10. A method as claimed in claim 8 wherein said cancer-associated marker protein is a modified MUC1, BRCA1, BRCA2, p53, c-myc, c-erb $\beta$ 2 or Ras protein associated with primary breast cancer.

25

11. A method as claimed in claim 8 wherein said cancer-associated marker protein is a modified MUC1, BRCA1, BRCA2, p53, c-myc, c-erb $\beta$ 2 or Ras protein associated with advanced breast cancer.

30

12. A method as claimed in claim 10 wherein said autoantibodies are obtainable from monocytes isolated from a patient with primary breast cancer.

35

13. A method as claimed in claim 11 wherein said autoantibodies are obtainable from monocytes isolated from a patient with advanced breast cancer.

5           14. A method as claimed in any preceding claim wherein said autoantibodies are produced by an immortalized cell-line.

10           15. A method as claimed in any preceding claim wherein said autoantibodies are produced by a hybridoma.

15           17. A method as claimed in any of claims 1 to 14 wherein said autoantibodies are polyclonal antibodies.

            15. A method as claimed in any of claims 1 to 15 wherein said autoantibodies are monoclonal antibodies.

20           18. A method as claimed in any preceding claim wherein said autoantibodies are immobilized on a solid surface.

25           19. A method as claimed in claim 18 wherein any complexes formed between said autoantibodies and any cancer-associated marker protein present in said sample are detected using secondary antibodies or autoantibodies specific for at least one epitope of said marker protein, said secondary autoantibodies carrying a detectable label.

30           20. A method as claimed in claim 18 wherein in addition to said sample a labeled cancer-associated marker protein is added carrying at least one epitope recognised by said autoantibodies.

35

21. Use of a method as claimed in claims 1 to 20 to screen for recurrence of cancer after a treatment, to monitor systemic therapies or to select therapies.

5           22. A diagnostic reagent which comprises mammalian autoantibodies with a specificity for at least one epitope of a mammalian cancer-associated marker protein.

10           23. A reagent as claimed in claim 22 wherein said autoantibodies are human autoantibodies and said marker protein is a human cancer-associated marker protein.

15           24. A reagent as claimed in claim 22 or 23 wherein said autoantibodies have specificity for at least one epitope of a cancer-associated marker protein associated with lymphomas, leukaemias, breast cancers, colorectal cancers, lung cancers, pancreatic  
20 cancers, prostate cancers, cervical cancers, ovarian cancers, endometrial cancers or cancers of the skin.

            25. A reagent as claimed in claim 24 wherein said autoantibody have specificity for at least one  
25 epitope of a breast cancer-associated marker protein.

            26. A reagent as claimed in claims 22 to 25 wherein said marker protein is a modified MUC1, BRCA1, BRCA2, p53, c-myc, c-erb $\beta$ 2 or Ras protein.

30           27. A reagent as claimed in claim 25 wherein said marker protein is a modified MUC1, BRCA1, BRCA2, p53, c-myc, c-erb $\beta$ 2 or Ras protein associated with primary breast cancer.

35

28. A reagent as claimed in claim 25 wherein said marker protein is a modified MUC1, BRCA1, BRCA2, p53, c-myc, c-erbβ2 or Ras protein associated with advanced breast cancer.

5

29. A reagent as claimed in claim 27 wherein said autoantibodies are obtainable from monocytes isolated from a patient with primary breast cancer.

10

30. A reagent as claimed in claim 28 wherein said autoantibodies are obtainable from monocytes isolated from a patient with advanced breast cancer.

15

31. An immortalized cell-line capable of producing autoantibodies directed against at least one epitope of a mammalian cancer-associated marker protein.

20

32. A cell line as claimed in claim 31 capable of producing autoantibodies directed against at least one epitope of a human cancer-associated marker protein.

25

33. An immortalized cell-line as claimed in claims 31 and 32 wherein said autoantibodies are directed against at least one epitope of a cancer-associated marker protein associated with lymphomas, leukaemias, breast cancers, colorectal cancers, lung cancers, pancreatic cancers, prostate cancers, cervical cancers, ovarian cancers, endometrial cancers or cancers of the skin.

30

34. An immortalised cell-line as claimed in claim 33 wherein said autoantibodies are directed against an epitope of breast cancer-associated marker

35

protein.

35. A cell line as claimed in claim 31 to 34  
wherein said autoantibodies are directed against a  
5 modified MUC1, BRCA1, BRCA2, p53, c-myc, c-erb $\beta$ 2 or  
Ras protein.

36. An immortalized cell-line as claimed in  
claim 34 wherein said autoantibodies are  
10 autoantibodies to a modified MUC1, BRCA1, BRCA2, c-  
myc, p53, c-erb $\beta$ 2 or Ras protein associated with  
primary breast cancer.

37. An immortalized cell-line as claimed in  
15 claim 34 wherein said autoantibodies are  
autoantibodies to a modified MUC1, BRCA1, BRCA2, c-  
myc, c-erb $\beta$ 2 or Ras protein associated with advanced  
breast cancer.

20 38. An immortalised cell line as claimed in  
claim 36 wherein said cell line is derived from  
monocytes of patients with primary breast cancer.

39. An immortalised cell line as claimed in  
25 claim 37 wherein said cell line is derived from  
monocytes of patients with advanced breast cancer.

40. A hybridoma capable of producing monoclonal  
autoantibodies directed against an epitope of a  
30 mammalian cancer-associated marker protein.

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41. A hybridoma as claimed in claim 40 wherein  
said monoclonal autoantibodies are directed against an  
epitope of a human cancer-associated marker protein.

42. A hybridoma as claimed in claims 40 and 41 wherein said autoantibodies are directed against a cancer-associated marker protein associated with lymphomas, leukaemias, breast cancers, colorectal  
5 cancers, lung cancers, pancreatic cancers, prostate cancers, cervical cancers, ovarian cancers, endometrial cancers or cancers of the skin..

43. A hybridoma as claimed in claim 42 wherein  
10 said autoantibodies are directed against a breast cancer-associated marker protein.

44. A hybridoma as claimed in claims 40 to 43 wherein said autoantibodies are directed against a  
15 modified MUC1, BRCA1, BRCA2, p53, c-myc, c-erb $\beta$ 2 or Ras protein.

45. A hybridoma as claimed in claim 43 wherein said autoantibodies are antibodies to a modified MUC1,  
20 BRCA1, BRCA2, p53, c-myc, c-erb $\beta$ 2 or Ras protein associated with primary breast cancer.

46. A hybridoma as claimed in claim 43 wherein said autoantibodies are antibodies to modified MUC1,  
25 BRCA1, BRCA2, p53, c-myc, c-erb $\beta$ 2 or Ras protein associated with advanced breast cancer.

47. A hybridoma as claimed in claim 45 wherein said hybridoma is derived from monocytes of patients  
30 with primary breast cancer.

48. A hybridoma as claimed in claim 46 wherein said hybridoma is derived from monocytes of patients with advanced breast cancer.

49. A kit for detecting a cancer-associated marker protein present in a bodily fluid of a mammal which kit comprises:

- 5           (a) mammalian autoantibodies directed against a cancer-associated marker protein from the same species as said autoantibodies; and
- 10           (b) means for detecting the formation of complexes between said autoantibodies and said cancer-associated marker protein.

50. A kit as claimed in claim 49 wherein said autoantibodies are immobilized on a solid surface.

15

51. A kit as claimed in claim 49 or 50 wherein said autoantibodies are human autoantibodies

20           52. A kit as claimed in claims 49 to 51 wherein said marker protein is a cancer-associated marker protein associated with lymphomas, leukaemias, breast cancers, colorectal cancers, lung cancers, pancreatic cancers, prostate cancers, cervical cancers, ovarian cancers, endometrial cancers or cancers of the skin.

25

53. A kit as claimed in claims 52 wherein said marker protein is a breast-cancer associated marker protein.

30

54. A kit as claimed in claims 49 to 53 wherein said marker protein is a modified MUC1, BRCA1, BRCA2, p53, c-myc, c-erb $\beta$ 2 or Ras protein.

35           55. A kit as claimed in claim 53 wherein said marker protein is a modified MUC1, BRCA1, BRCA2, c-

myc, p53, c-erb $\beta$ 2 or Ras protein associated with primary breast cancer.

5        56. A kit as claimed in claim 53 wherein said marker protein is a modified MUC1, BRCA1, BRCA2, p53, c-myc, c-erb $\beta$ 2 or Ras protein associated with advanced breast cancer.

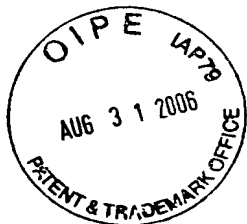
10       57. A method for detecting a cancer-associated marker protein present in a bodily fluid of a mammal substantially as described herein with reference to the accompanying examples.

15       58. A kit for detecting a cancer-associated marker protein present in a bodily fluid of a mammal substantially as described herein with reference to the accompanying examples.

20       59. A diagnostic reagent substantially as described herein with reference to the accompanying examples.

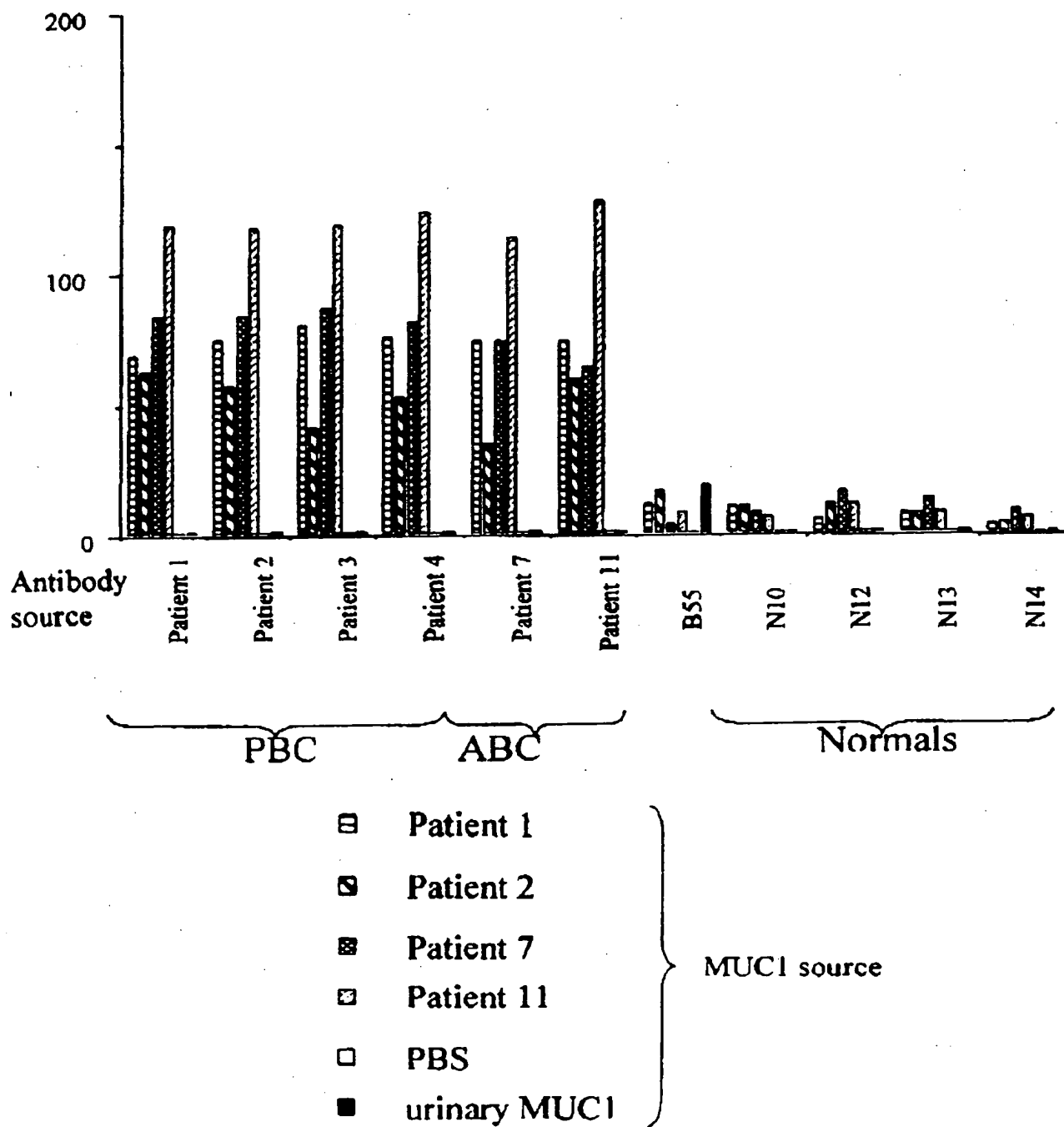
25       60. A cell-line capable of producing autoantibodies directed against one or more epitopes of a cancer-associated marker protein substantially as described herein with reference to the accompanying examples.





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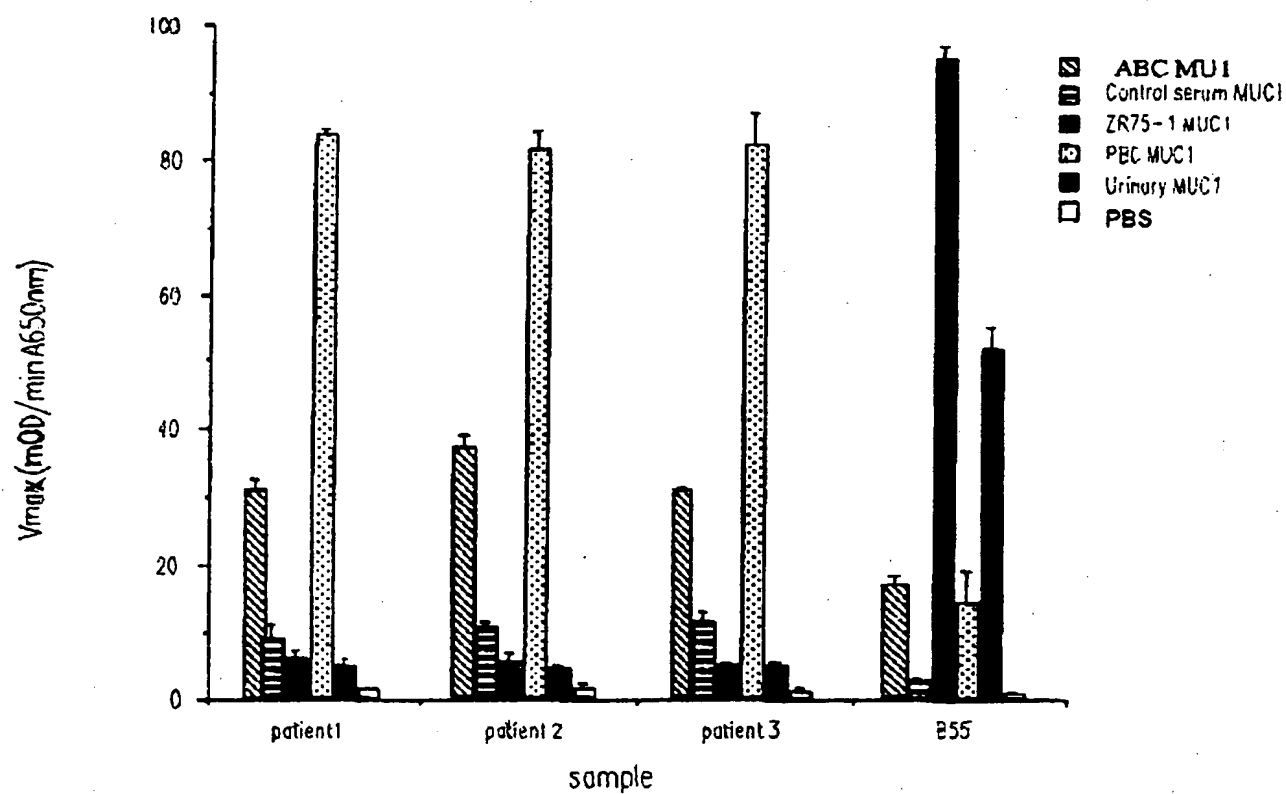
FIG. 1.



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FIG. 2.

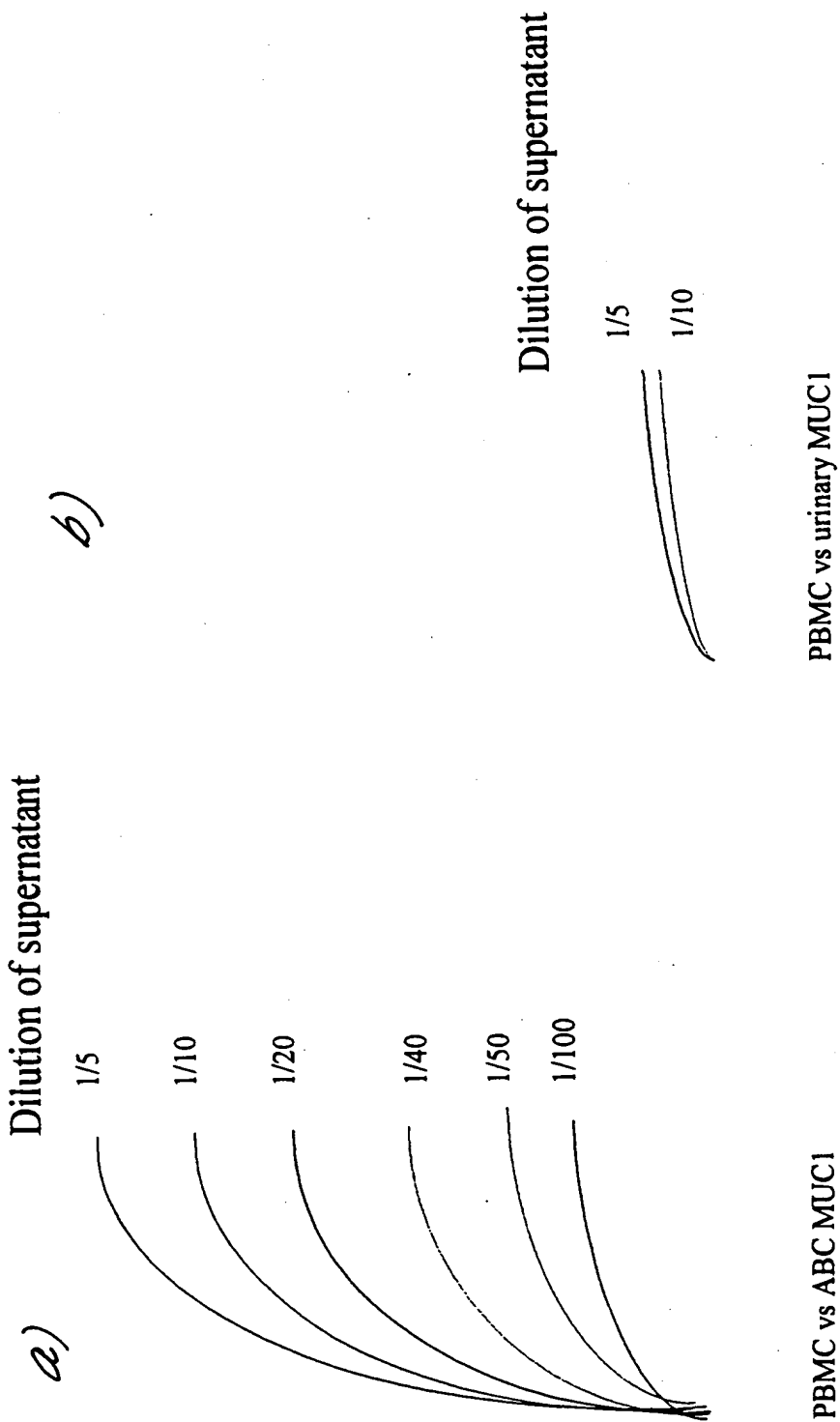




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FIG. 3.

SPR results depicting the rate of binding of PBMC supernatant to ABC and urinary MUC1



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FIGURE 4

TAPPAHGV T\* SAPDTRPAGST\* APPA

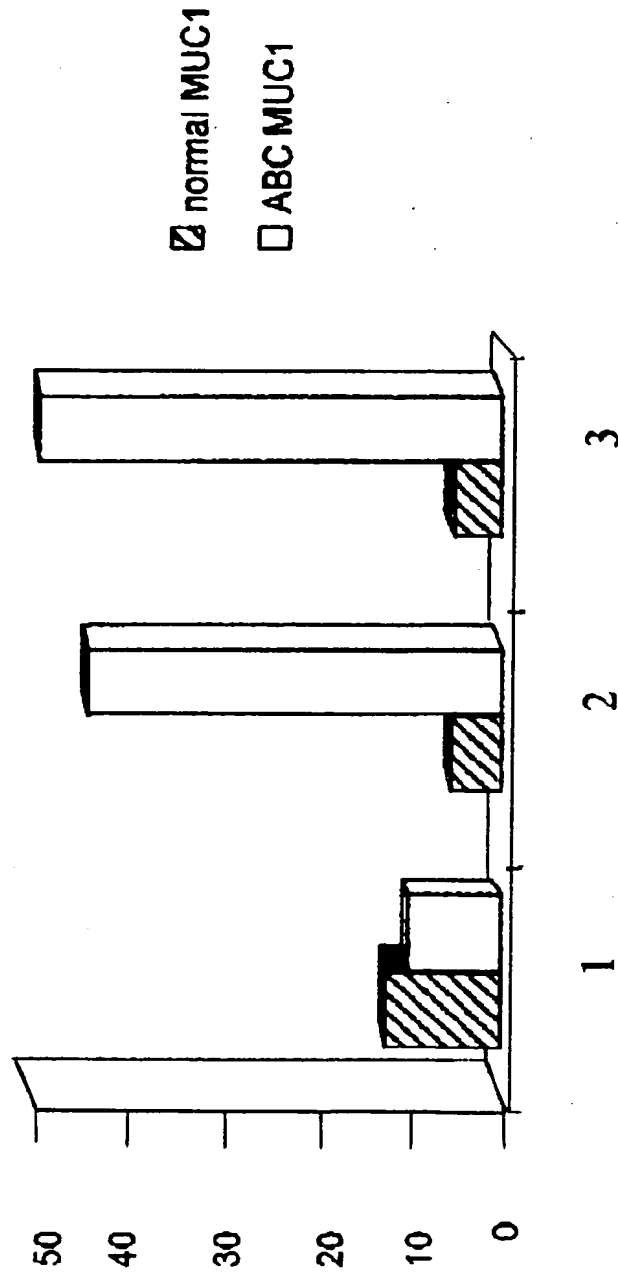
T\* are O-glycosylated with N-acetyl-galactosamine

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FIG. 5.

Sandwich ELISA to detect MUC1 (normal and tumour associated).



Sandwiches of :- 1 = C595/Biotinylated C595

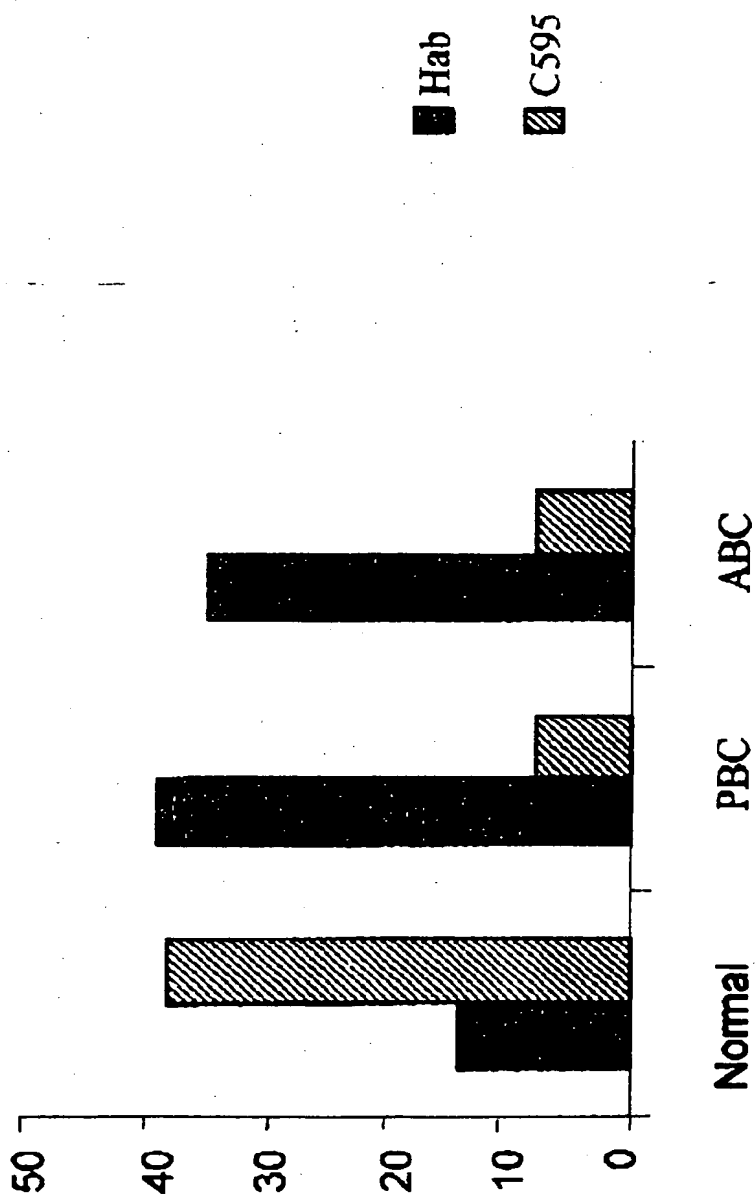
2 = PBC culture sup. }

3 = ABC culture sup. } / Biotinylated TAP2 purified ABC serum antibodies

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FIG. 6.

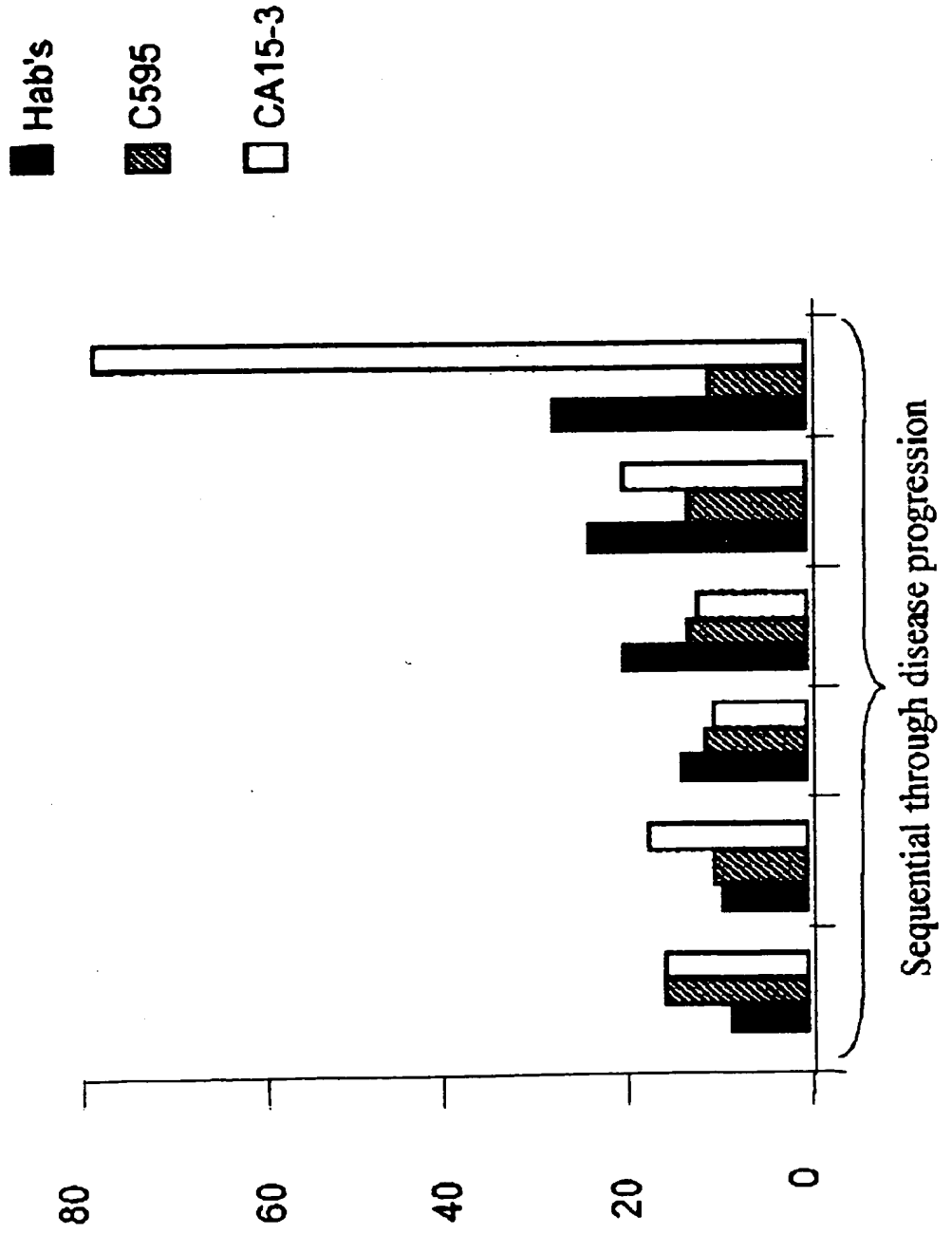
Sandwich ELISA's performed on normal and cancer samples



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FIG. 7.

Sandwich ELISA's performed on sequential samples from a patient with metastatic breast cancer.



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